Flavoenzymes

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Published in:
Trends in Biochemical Sciences

DOI:
10.1016/S0968-0004(99)01533-9

Publication date:
2000

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Flavoenzymes: diverse catalysts with recurrent features

Marco W. Fraaije and Andrea Mattevi

Many biochemical processes exploit the extraordinary versatility of flavoenzymes and their flavin cofactors. Flavoproteins are now known to have a variety of folding topologies but a careful examination of their structures suggests that there are recurrent features in their catalytic apparatus. The flavoenzymes that catalyse dehydrogenation reactions share a few invariant features in the hydrogen-bond interactions between their protein and flavin constituents. Similarly, the positioning of the reactive part of the substrate with respect to the cofactor is generally conserved. Modulation of substrate and cofactor reactivity and exact positioning of the substrate are key elements in the mode of action of these enzymes.

FLAVOENZYMES HAVE THE unique ability to catalyse a wide range of biochemical reactions. They are involved in the dehydrogenation of a variety of metabolites, in one- and two-electron transfer from and to redox centres, in light emission, in the activation of oxygen for oxidation and hydroxylation reactions[1]. Because of the extraordinary spectroscopic properties of the flavin cofactor, flavoproteins are perfectly suited to detailed enzymological studies[1]. As a result, they have emerged as one of the best-studied enzyme families.

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Our understanding of flavin chemistry has been augmented in the recent years by the wealth of information obtained from structural studies of these proteins. At present, the Protein Data Bank (PDB)[2] contains about 200 entries for flavin adenine dinucleotide (FAD)-dependent and flavin mononucleotide (FMN)-dependent proteins. These structures display a number of folding architectures, ranging from the frequent $(\alpha\beta)_8$ barrel to unique topologies, such as the $\alpha+\beta$ structure of acylCoA dehydrogenases[3].

The distribution of these folds does not correlate with function. Topologically similar flavoenzymes can catalyse different reactions, whereas proteins performing similar functions can have dissimilar folding architectures. For example, flavocytochrome b_2 (Ref. 4) and D-amino acid oxidase[5,6] display completely different topologies, even though they catalyse a similar chemical reaction (Table 1). Conversely, functionally dissimilar enzymes, such as oxidases (D-amino acid oxidase[5,6] and cholesterol oxidase[7]), hydroxylases (*p*-hydroxybenzoate hydroxylase[8]) and oxidoreductases (fumarate reductase and related enzymes[9,10]), share a topologically similar FAD-binding domain. In this respect, flavoproteins are perfectly in line with the finding of Martin *et al.*[11] that there is little relationship between folding topology and function.

The catalytic power of an enzyme lies in its active site, in which the atoms are positioned to allow substrate binding and to stabilize reaction intermediates. It is well known that structurally dissimilar proteins can employ the same active-site geometry and catalytic devices to perform similar functions. This notion is beautifully exemplified by the so-called catalytic triad present in a vast number of otherwise unrelated families of hydrolase enzymes (recently reviewed in Ref. 12).

On this basis, we have examined the three-dimensional structures of flavoenzymes that are available through the PDB and searched for recurrent features in their catalytic apparatus. Our analysis is focused on the group of flavoproteins that perform a dehydrogenation reaction[13]. This involves the rupture of a kinetically stable C–H bond, coupled to the transfer of two electrons to the flavin (Fig. 1). Throughout this article, we shall call the carbon atom involved

0968–0004/00/$ – See front matter PII: S0968-0004(99)01533-9

Table 1. Binding features of the isoalloxazine ring in flavin-dependent enzymes

Flavoenzyme[a]	Ref.	Cofactor[b]	Folding topology	Protein residue contacting N–C2=O2	H-bond donor to N5			
					H-bond partner	Distance (Å)	Angle with N10··N5 (°)	Flavin side
Old yellow enzyme (1OYB)	36	FMN *si*	$(\alpha/\beta)_8$ barrel	R243	N-T37	2.8	151	*re*
Flavocytochrome b_2 (1FCB)	4	FMN *si*	$(\alpha/\beta)_8$ barrel	K349	N-A198	3.0	139	*re*
Trimethylamine dehydrogenase (2TMD)	16	FMN *si*	$(\alpha/\beta)_8$ barrel	R222	N-C30	3.0	116	*re*
Dihydroorotate dehydrogenase (2DOR)	37	FMN *si*	$(\alpha/\beta)_8$ barrel	K164	Nζ-K43	3.3	170	*re*
Glycolate oxidase[c] (1GOX)	35	FMN *si*	$(\alpha/\beta)_8$ barrel	K230				
D-amino acid oxidase (1DDO)	5	FAD *re*	PHBH fold	α Helix	N-A49	3.3	125	*si*
Cholesterol oxidase (1COY)	38	FAD *re*	PHBH fold	α Helix	N-G120	3.4	129	*si*
Polyamine oxidase[d] (1B37)	15	FAD *re*	PHBH fold	α Helix	H_2O[d]	3.0	152	*si*
Medium-chain acylCoA dehydrogenase (3MDE)	3	FAD *re*	Three domains α+β	N-V135 N-T136	Oγ -T168	2.9	170	*si*
NAD(P)H quinone reductase (1QRD)	39	FAD *si*	Flavodoxin like	N-G150 N-G149	N-W105	3.1	145	*re*
Vanillyl-alcohol oxidase[c] (2VAO)	26	FAD *si*	Two domains α+β	R504				
MurB[e] (2MBR)	40	FAD *si*	Two domains α+β	R159	Nη2-R214	2.8	153	*re*
Flavocytochrome c_3 (1QJD)	24	FAD *re*	Multidomain, dinucleotide-binding fold	α Helix	N-A169	3.3	158	*si*

[a]Name and PDB entry for the flavoenzyme structures. When available, structures of enzymes in complex with active-site ligands were used. Glucose oxidase[33] and *p*-cresol methylhydroxylase[34] are not listed, as they share considerable homology and essentially identical flavin sites with cholesterol oxidase[7] and vanillyl-alcohol oxidase[26], respectively. Flavocytochrome c_3 (Ref. 24) has been chosen as the representative protein of the succinate dehydrogenase family of oxidoreductases, which includes L-aspartate oxidase[10] and fumarate reductase[9]. PHBH fold indicates the folding topology first observed in *p*-hydroxybenzoate hydroxylase[8].
[b]Type of cofactor and position of the substrate-binding site with respect to the isoalloxazine ring. When viewing the *re* face of the flavin ring, with the ribityl chain pointing upwards, the pyrimidine ring is on the left; when viewing the *si* face, the pyrimidine ring is on the right (see Fig. 1).
[c]In glycolate oxidase, the N5 atom is not involved in any H bond, because of its location at 3.4 Å from O-T345. Similarly, in vanillyl-alcohol oxidase, Oδ2-D170 is the atom closest to N5 (3.7 Å).
[d]The ordered water molecule that is H-bonded to N5 of polyamine oxidase also interacts with Nζ-K300.
[e]MurB is the acronym of UDP-*N*-acetyl*enol*pyruvoylglucosamine reductase.

in this C–H bond the site of oxidative attack. The reviewed enzymes display various folding topologies and are either FMN- or FAD-dependent proteins (Table 1). Despite such a structural diversity, they have a few remarkably similar properties in their mode of binding both flavin and substrate.

The flavin-binding site

The tricyclic isoalloxazine ring system is the reactive part of the flavin (Fig. 1). It is an amphipathic molecule formed by the fusion of the hydrophobic dimethylbenzene moiety with the hydrophilic pyrimidine ring. The redox potential for the two-electron reduction of the flavin is about −200 mV. However, this value can greatly vary in flavoenzymes, spanning a range from approximately −400 mV to +60 mV[13,14]. In general, the proximity of a positive charge is thought to increase the redox potential, whereas a negative charge or a hydrophobic environment are expected to lower it[1,13]. Remarkably, a few flavoenzymes have a covalently bound FAD molecule. Site-directed mutagenesis studies suggest that such a covalent interaction could enhance the oxidative power of the flavin[14]. These observations highlight the crucial role that is played by the protein milieu in fine-tuning the chemical properties of the flavin.

The isoalloxazine can adopt conformations that deviate significantly from exact planarity (e.g. polyamine oxidase[15], cholesterol oxidase[7] and trimethylamine dehydrogenase[16]). However, independent of the degree of planarity, the ability of the flavin to form hydrogen bonds appears to be fully satisfied in all enzymes under investigation. In all the structures examined (Table 1), the N1–C2=O2 locus (Fig. 1) is always in contact (<3.5 Å distance) with a positively charged entity. This can be either fully charged, such as a Lys or Arg side chain, or partially charged, such as the N terminus of an α helix or a cluster of peptide nitrogens (Table 1). A positive charge at this location is functionally relevant because it can stabilize the anionic form of the reduced flavin (Fig. 1) and increase the cofactor's redox potential[13]. Moreover, in dihydroorotate dehydrogenase[17] and trimethylamine dehydrogenase[18], replacing the positively charged residue close to N1 with a neutral side chain resulted in an inactive enzyme that was unable to bind to FMN. Thus, the recurrent presence of a positively charged

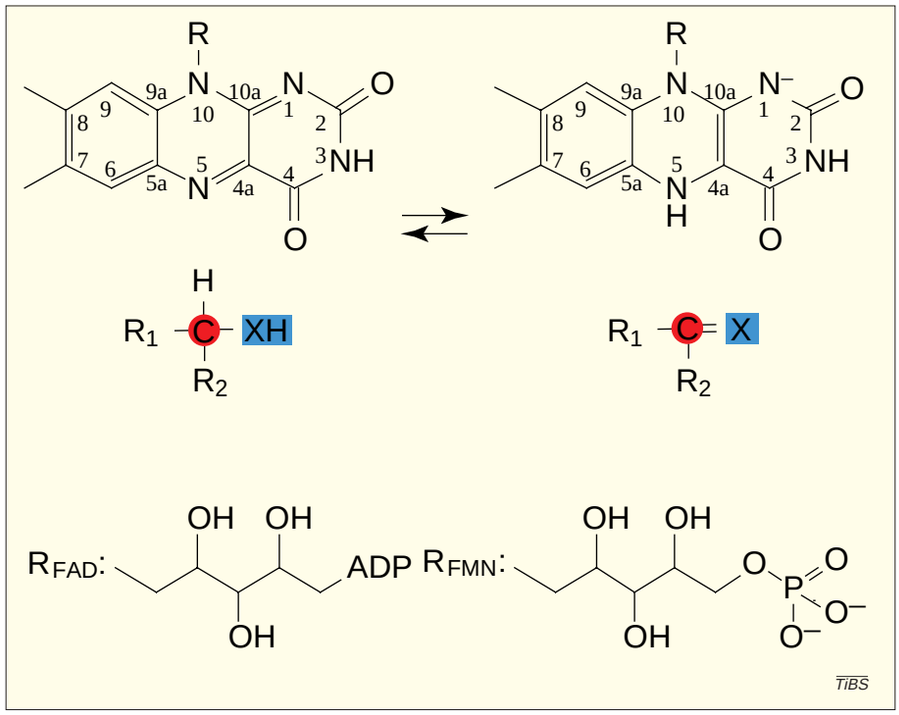


Figure 1

General scheme of a flavin-dependent dehydrogenation reaction. The carbon atom that undergoes the rupture of a C–H bond with transfer of two electrons to the flavin represents the site of oxidative attack (red circle). The 'activating' X group (blue square) facilitates the dehydrogenation reaction. The atomic positions discussed in the text are numbered. The isoalloxazine system is shown with the *si* side facing the viewer (see Table 1). R_1 and R_2 indicate unspecific substituents of the CH atom undergoing oxidation.

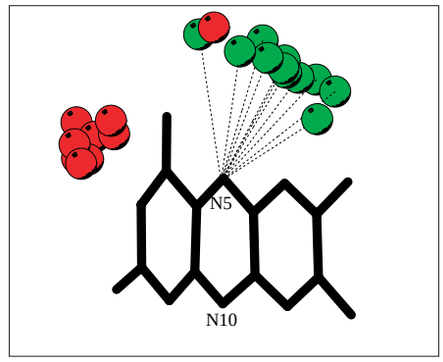


Figure 2

Flavin-N5–protein interactions and substrate binding in flavoenzyme structures. The image was generated as follows. The isoalloxazine atoms of each enzyme were superimposed onto the flavin ring of flavocytochrome b_2 and the resulting rotation and translation operators were applied to each model. Next, the atomic coordinates of those enzymes that bind the substrate on the flavin *re* side (see Table 1) were transformed by an operation corresponding to a mirror coplanar to the isoalloxazine ring. The mirror operation was applied in order to facilitate the visualization of the features conserved across flavoenzyme structures. The isoalloxazine rings of trimethylamine dehydrogenase, polyamine oxidase and cholesterol oxidase are bent, and so only the C4a, N5, C5a, C9a, N10 and C10a atoms were used in the superposition calculations for these enzymes. The protein atoms that are engaged in a hydrogen bond with the N5 atom (Table 1) are in green. The carbon atoms representing the site of oxidative attack, as deduced from the structures of enzyme complexes (Table 2), are in red. With the exception of cholesterol oxidase, all these sites cluster in a well-defined position in front of N5.

group neighbouring the N1–C2=O2 locus simultaneously serves two purposes: to favour flavin binding and to regulate the redox properties of the cofactor.

A second recurrent feature emerging from the analysis of the isoalloxazine–protein interactions concerns the N5 locus (Fig. 1). In most enzymes, this atom is within hydrogen-bond distance from a hydrogen-bond donor, typically a backbone or side-chain nitrogen atom. The bond is present both in enzymes that bind to their substrate on the flavin *si* face or *re* side (Fig. 2). Remarkably, the stereochemistry of this interaction is highly conserved: the hydrogen-bond donor is located on the flavin side opposite to that facing the substrate, the angle between N10, N5 and the hydrogen-bond donor ranging from 116° to 170° (Table 1, Figs 2,3). N5 takes part directly in substrate dehydrogenation and any interaction involving this atom will affect catalysis. The proximity of a hydrogen-bond donor is generally expected to increase the oxidative power of the cofactor[13]. However, the particular hydrogen bond at N5 might have a more subtle effect. Upon reduction, N5 becomes protonated and so the hydrogen-bond interaction might become energetically less favourable in the reduced than in the oxidized state. Noticeably, vanillyl-alcohol oxidase[14] and glycolate oxidase[19], which are unusual because they lack the N5 hydrogen bond, exhibit a relatively high (>−25 mV) redox potential. Further studies are needed to clarify the exact role of the frequently observed hydrogen bond at the N5 locus.

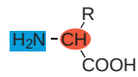
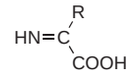
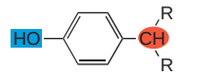
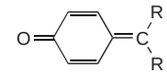
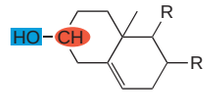
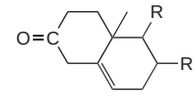
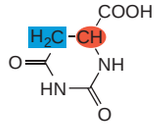
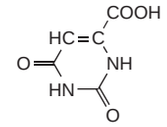
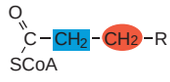
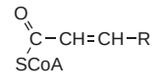
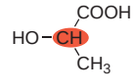
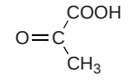
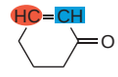
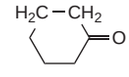
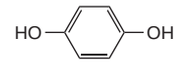
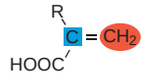
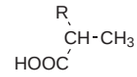
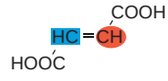
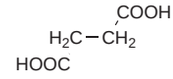
The substrate-binding site

Owing to the inherent instability of enzyme–substrate complexes, their three-dimensional structures are difficult to analyse by X-ray crystallography. However, the stereochemistry of substrate binding can be inferred from the structures of the enzymes in complex with reaction products or competitive inhibitors that are sufficiently similar to the substrate. The proteins under investigation act on substrates that vary greatly in both chemical nature (amines, alcohols, fatty acids, hydroxy and amino acids) and size [ranging from bulky cholesterol to small lactate (Tables 1, 2)].

Despite this diversity, examination of the enzyme complexes reveals some common features. When the isoalloxazine rings of these structures are superimposed, a striking similarity emerges in the position of the ligand atom that mimics the substrate atom being dehydrogenated by the flavin. As inferred from the crystal structures of enzyme complexes, the site of oxidative attack (Fig. 1) typically binds in front of the flavin at 3.5 Å distance from N5, defining an angle with the N5–N10 atoms in the narrow range of 96–117° (Figs 2,3; Table 2).

To visualize this similarity, it is instructive to project the substrate atoms onto the plane defined by the isoalloxazine ring. This shows that the projected position of the site of oxidative attack falls in a well-defined location. More precisely, the projected position is offset by 0.2–1.1 Å from the line connecting N5 to N10 (distance *a* in Fig. 3). The offset is in the direction of the O4 locus and so, the projected position invariably falls between the two lines defined by the C4a–C10a and N5–N10 atoms, respectively. Such a detailed level of conservation is truly remarkable. It shows how carefully flavoenzyme active

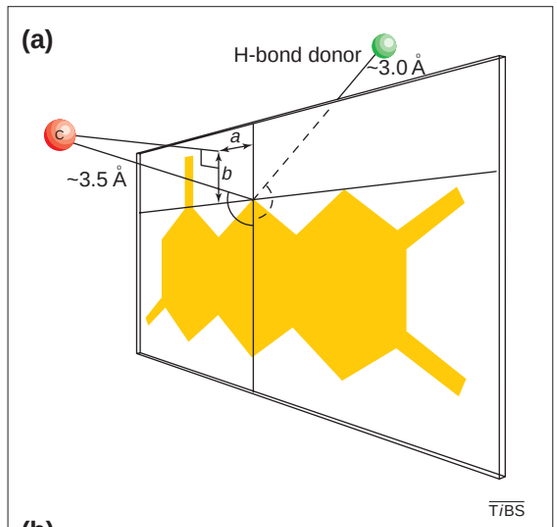
Table 2. Stereochemistry of substrate binding in flavoenzyme structures

Enzyme	Substrate[a]	Product	Substrate activation X	Distance[b] (Å) N5⋯CH	Angle[b] (°) N10⋯N5⋯CH	Refs
D-amino acid oxidase			Deprotonated amino group	3.8	110	5,27
Vanillyl-alcohol oxidase			Phenolate anion stabilization	3.3	117	26
Cholesterol oxidase			Deprotonation of hydroxyl group by His	3.7	162	7
Dihydroorotate dehydrogenase			α-Proton abstraction by Cys	3.5	96	17,22
AcylCoA dehydrogenase			α-Proton abstraction by Glu	3.0	106	20,21
Flavocytochrome b_2			Unresolved[c]	3.7	104	
Old yellow enzyme[d]			Proton donation by Tyr side chain	3.5	101	25
NADPH quinone reductase[d]			Unresolved	3.5	103	
UDP-*N*-acetyl*enol*pyruvoyl-glucosamine reductase[d]			Proton donation to Cα by Ser side chain	3.1	108	40
Flavocytochrome c_3[d]			Proton donation by Arg side chain	3.4	98	24

[a]The dotted circles and squares outline the site of oxidative attack and the group facilitating the formation of the double bond in the oxidized product, respectively.
[b]The stereochemical parameters in substrate binding were deduced from the available structures of enzymes in complex with substrate analogues.The position of the CH group representing the site of oxidative attack were measured based on the following atoms (see Table 1 for PDB entries): Cα of imino-Trp in complex with reduced D-amino acid oxidase; C7 of isoeugenol bound to vanillyl-alcohol oxidase; C3 of dehydroisoandrosterone bound to cholesterol oxidase; C6 of orotate bound to dihydroorotate dehydrogenase; C3 of octanoylCoA bound to medium chain acylCoA dehydrogenase; C2 of pyruvate bound to flavocytochrome b_2; C2 of *p*-hydroxybenzaldehyde bound to old yellow enzyme; C2 of duroquinone in complex with NADPH-quinone reductase; EC3 of *enol*pyruvoyl-UDP-*N*-acetyl-glucosamine in complex with UDP-*N*-acetyl*enol*pyruvoylglucosamine reductase and, C3 of malic acid bound to flavocytochrome c_3. This analysis does not include the complexes of glycolate oxidase with 3-decyl-2,5-dioxo-4-hydroxy-3-pyrroline and 4-carboxy-5-(1-pentyl)hexylsulphanyl-1,2,3-triazole because these inhibitors bear little resemblance to the substrate[35]. Similarly, the complex between polyamine oxidase and MDL72527 (Ref. 15) was not considered, because it does not allow an unambiguous assignment of the position of the site of oxidative attack.
[c]The mechanism of the lactate dehydrogenation reaction catalysed by flavocytochrome b_2 is controversial[30]. In the assumption of a carbanion mechanism, His373 would abstract the substrate α-proton. Alternatively, in the hypothesis of a hydride transfer mechanism, His373 is supposed to deprotonate the substrate hydroxyl group.
[d]Old yellow enzyme, NADPH quinone reductase, UDP-*N*-acetyl*enol*pyruvoylglucosamine reductase and flavocytochrome c_3 catalyse a 'hydrogenation' (i.e. substrate reduction) rather than a dehydrogenation reaction.

centres are designed and optimized to achieve a stereochemically precise coordination between the flavin and the reactive part of the substrate.

Cholesterol oxidase exhibits a binding mode of the substrate that deviates significantly from the above-described stereochemistry[7] (Fig. 2), as inferred from the structure of the enzyme–product complex. However, this oxidase has a dual activity; it catalyses substrate oxidation followed by isomerization. The observed



(b)

Enzyme	*a* (Å)	*b* (Å)
D-amino acid oxidase	1.0	1.4
Vanillyl-alcohol oxidase	0.2	1.4
Dihydroorotate dehydrogenase	0.5	0.8
Medium-chain acylCoA dehydrogenase	0.7	0.8
Flavocytochrome b_2	0.6	0.8
Old yellow enzyme	1.1	0.7
NADPH quinone reductase	0.4	0.9
UDP-*N*-acetyl*enol*pyruvoyl-glucosamine reductase	0.3	0.7
Flavocytochrome c_3	0.7	0.5

Figure 3

(a) Recurrent features in flavin and substrate orientation (Tables 1,2). The hydrogen-bond distance between the hydrogen-bond donor (green) provided by the protein and N5 is ~3.0 Å. The CH group (red) being oxidized by the protein binds in front of the flavin, at a distance of ~3.5 Å distance from N5. The angles defined by the atoms N10–N5–hydrogen-bond donor and N10–N5–C are listed in Tables 1 and 2, respectively. The level of conservation in the positioning of the site of oxidative attack can be seen by looking at its projection onto the flavin plane. The projected position falls in a location defined by the parameters *a* and *b*, which describe the distance from the lines connecting N5 to N10 and C4 to C6, respectively. The observed *a* and *b* distances are given in the table in **(b)**.

binding mode thus might mimic the one required for the isomerization rather than the oxidation reaction. Alternatively, it might genuinely differ from that observed in other flavin-dependent oxidases.

The activating group

Completion of the dehydrogenation reaction requires the formation of a double bond in the oxidized product (Fig. 1). This is brought about by the group adjacent to the site of oxidative attack. Flavoenzymes are able to modulate the reactivity of this sort of 'activating group', facilitating the dehydrogenation reaction. In a few cases, the structural elements with this modulating function have been analysed in detail. Several flavoenzymes catalyse an α,β dehydrogenation. In this reaction, a carbon atom in the α position with respect to a carboxylate or carbonyl group is deprotonated by an active-site base to allow formation of an α,β double bond.

Enzymes that catalyse a reaction of this kind typically establish a network of hydrogen bonds with the substrate carbonyl or carboxylate oxygen. These interactions are crucial in that they lower the pK_a of the α carbon to make proton abstraction by a base feasible. A beautiful example of this type of modulation of substrate reactivity is provided by acylCoA dehydrogenases[20] (Table 2). These enzymes shift the pK_a of the acylCoA α carbon by 9–13 units using hydrogen bonds between the substrate carbonyl oxygen with the 2′-hydroxyl group of FAD and a backbone nitrogen atom. Such a drastic pK_a change allows a Glu side chain to act as the base that abstracts the proton from the substrate α carbon, facilitating the oxidation reaction[21].

A similar mechanism is employed by dihydroorotate dehydrogenase. In this enzyme, the substrate carbonyl group interacts with two Asn side chains, which increase the acidity of the adjacent carbon, facilitating its deprotonation by a Cys residue[17,22]. An analogous catalytic strategy is exhibited by UDP–*N*-acetyl*enol*pyruvoylglucosamine reductase[23], flavocytochrome c_3 (Ref. 24) and the old yellow enzyme[25], although these proteins catalyse the reaction in the opposite direction (a hydrogenation rather than a dehydrogenation reaction). In these enzymes, an active-site residue (Ser, Arg and Tyr, respectively) donates a proton to the substrate α carbon, coupled to reduction of the adjacent carbon atom by the flavin.

Vanillyl-alcohol oxidase displays another mechanism of substrate activation: the active-site cavity shifts the pK_a of the substrate, which is preferentially bound by the protein in the phenolate anionic form. In this way, the enzyme facilitates formation of the quinone methide intermediate, produced by substrate oxidation[26]. The intermediate is then hydrated in subsequent steps of the reaction. Although it is less well known, D-amino acid oxidase[5,27] is also thought to promote catalysis by acting on the protonation state of the substrate. This oxidase probably stabilizes the deprotonated form of the substrate amino group, thereby facilitating formation of the imino acid product.

There is an evident functional analogy between the cofactor- and substrate-binding sites. Neither has a passive role in ligand recognition but instead are both active in modulating the chemical properties of the bound molecule. The protein milieu is crucial in that it fine-tunes the redox properties of the flavin and enhances the reactivity of the substrate towards dehydrogenation.

Protecting the substrate from the solvent

Another notable feature shared by flavin-dependent dehydrogenases is the accessibility of the active site. In all the available structures of enzyme complexes, it can be seen that the reactive part of the substrate invariably binds in a buried site located underneath the protein surface. This feature implies that catalysis takes place in a solvent-protected environment. Flavoenzymes are equipped with ingenious devices to open the active centre for substrate admission. In several cases, a mobile loop (D-amino acid oxidase[5], cholesterol oxidase[7] and dihydroorotate dehydrogenase[22]) or side chain (UDP–*N*-acetyl*enol*pyruvoylglucosamine reductase[28]) acts as a gate, closing and opening the active site. Likewise, a flexible domain can be used to fulfil this role, as observed in flavocytochrome c_3 and related enzymes of the succinate-dehydrogenase family[24]. Conversely, polyamine oxidase[15] and acylCoA dehydrogenases[3] use a different technique to shield the substrate. In both cases, the exact match of the substrate with a narrow active-site channel causes the solvent to be expelled upon substrate binding.

Shielding the substrate from solvent is also a common phenomenon among the NAD(P)H-dependent dehydrogenases[29]. Protection from solvent seems to be a prerequisite for catalysing a dehydrogenation reaction, regardless of the type of cofactor employed by the enzyme.

Implications for the catalytic mechanism

A comparison of the catalytic sites of flavoenzymes catalysing dehydrogenation

reactions has led to the recognition of several structural relationships. These proteins appear to have adopted a sort of 'two-handle' strategy to perform their catalytic function. The site of oxidative attack represents the first 'handle': flavoproteins bind the substrate CH group undergoing oxidation in a precisely defined position in front of the N5–C4a locus of the isoalloxazine ring (Fig. 3). The second 'handle' is given by the 'activating group', which is involved in the formation of the double bond of the dehydrogenated product. The protein actively modulates the reactivity of this group, often by affecting its protonation state.

Three additional features augment the catalytic effectiveness of such a two-handle strategy. First, the protein–flavin interactions are effective at fine-tuning the cofactor's redox properties. Second, the conserved hydrogen-bond interactions at the N1 and N5 loci can regulate the ability of the flavin to carry out the two-electron oxidation of the substrate. Finally, the substrate and the residues directly involved in catalysis are invariably shielded from the solvent, implying that catalysis takes place in a protected and highly controlled environment.

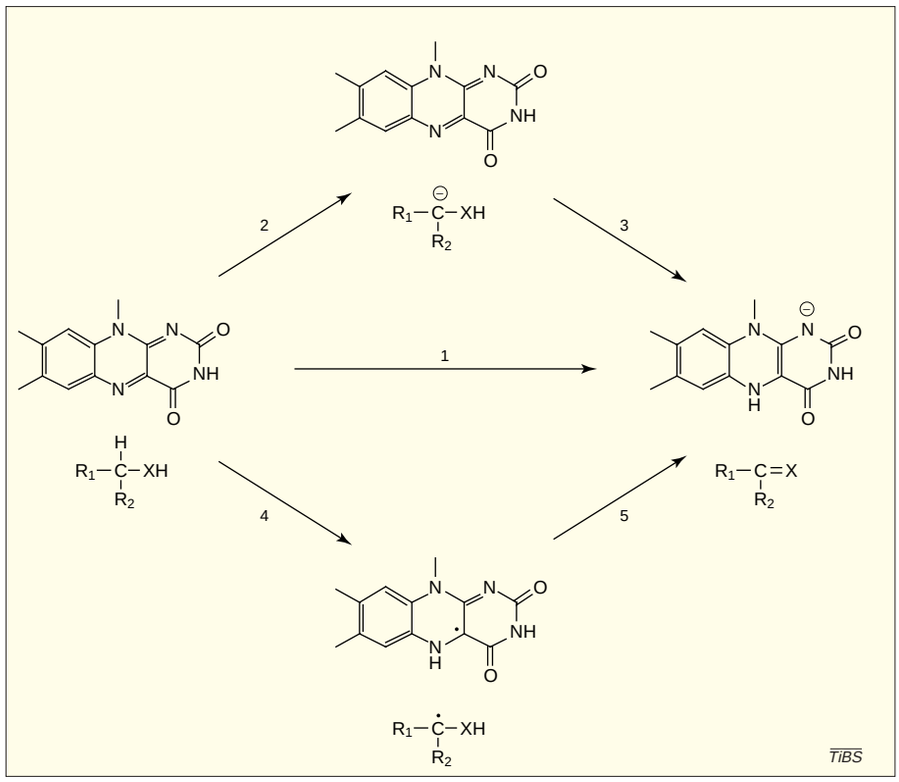


Figure 4

Schematic view of different mechanisms for flavin-catalysed substrate-dehydrogenation reactions – direct hydride transfer (1), the carbanion mechanism (2,3) and the radical mechanism (4,5). R_1 and R_2 indicate unspecific substituents of the CH atom undergoing oxidation, and X is the activating group.

The presence of these structural relationships raises the question of their mechanistic implications. A fundamental problem in flavoenzymology concerns the detailed mechanism of the dehydrogenation step: how do flavoenzymes catalyse the rupture of the C–H bond at the site of oxidative attack with transfer of two electrons to the flavin? Several hypotheses have been put forward[13,27,30,31] (Fig. 4):

(1) hydride transfer, which involves the direct transfer of a hydride anion from the substrate C–H group to the flavin N5 position;

(2) the radical mechanism, in which rupture of the C–H bond occurs by the removal of a hydrogen atom from the substrate carbon with formation of a radical pair that collapses to produce the reduced flavin and oxidized product;

(3) the carbanion mechanism, in which an active-site base removes a proton from the substrate C–H group, thus producing a carbanion that donates two electrons to the flavin either directly or via a covalent intermediate.

These mechanistic problems cannot be solved solely on the basis of structural data. However, it seems that all these mechanistic proposals require, at least to some extent, juxtaposition between the flavin N5–C4a locus and the reactive CH group of the substrate. Thus, generally speaking, the stereochemically conserved proximity between the flavin and the site of oxidative attack does not necessarily indicate the existence of just one conserved mechanism for flavin-mediated dehydrogenation. Indeed, although many of the reviewed enzymes are proposed to function via hydride transfer, there is no general consensus about the exact mechanism for some of them, flavocytochrome b_2 and D-amino acid oxidase being the most controversial cases[13,27,30].

On the other hand, the stereochemical principles underlying the mutual interactions between the substrate CH group and the flavin are surprisingly well conserved and cannot be neglected. They represent a validation test for the plausibility of any proposed mechanism, which must be compatible with the observed stereochemistry of substrate binding. An important challenge for future flavoenzymological studies will be defining the exact stereochemical requirements of each of the proposed mechanisms, to evaluate their compatibility with the three-dimensional structures. In this regard, significant insight will be gained from the examination of flavoenzyme structures refined at atomic resolution, which will become available thanks to the power of the newest synchrotron X-ray diffraction beam lines. These structures will provide the framework for quantum-mechanical calculations and molecular simulations[32]. The integration of these data with those derived from classical enzymological analyses will ultimately solve this mechanistic riddle.

Acknowledgements

We thank D.E. Edmondson, B. Curti, W.J.H. Van Berkel and F. Lederer for helpful suggestions. The authors' work has been supported by grants from Ministero della Università e Ricerca Scientifica e Tecnologica (Progetti 'Biologia Strutturale', 'Biosintesi del NAD: studi biochimici, biologia strutturale e sviluppo razionale di farmaci') and Consiglio Nazionale delle Ricerche (Target Project 'Biotecnologie'). M.W.F. was supported by a long-term EMBO fellowship. Owing to space limitations, many articles describing enzymes included in the analysis had to be omitted. We apologize to the authors of these articles.

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